

Unique Cholesteryl Glucosides in *Helicobacter pylori*: Composition and Structural Analysis

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A chloroform-methanol-extracted lipid of *Helicobacter pylori* was studied. Three kinds of glycolipids, accounting for about 25% (wt/wt) of the total lipid, were detected and identified to be cholesteryl glucosides. The structures of two of them were determined to be cholesteryl- α -D-glucopyranoside and cholesteryl-6-*O*-tetradecanoyl- α -D-glucopyranoside, and the plausible structure of the third one was identified as cholesteryl-6-*O*-phosphatidyl- α -D-glucopyranoside. Cholesteryl glucosides are very rare in animals and bacteria. Furthermore, those in *H. pylori* had an α -glycosidic linkage, which is rather unusual for natural glycosides, and a phosphate-linked cholesteryl glycoside like the cholesteryl-6-*O*-phosphatidyl- α -D-glucopyranoside has not been reported previously. As the cholesterol glucosides were detected in strains obtained from diverse geographical locations, the presence of cholesteryl glucosides in *H. pylori* is a very unique and a characteristic feature of the species. These findings add a new facet to the physiology and biochemistry, especially the cholesterol and glucose metabolism, of *H. pylori*. Furthermore, the cholesteryl glucosides of *H. pylori* showed hemolytic activities.

Helicobacter pylori is a curved gram-negative bacterium which was first reported in 1983 (21, 35). The organism is now recognized as a primary cause of active chronic gastritis and is reported to be associated with peptic ulcer disease, gastric adenocarcinoma, and low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue (4, 14, 36).

The organism can colonize in and under the mucus layer of the human gastric mucosa without the copresence of other bacterial species. It, living in the unique gastric environment, is likely to have features distinct from other bacteria. Furthermore, it has been found to possess a unique urease having a very low K_m value, which hydrolyzes urea efficiently even at the low urea concentration found in the gastric mucosa (13, 24).

On the other hand, the lipid composition of microorganisms can adapt in response to alterations of environmental conditions (15, 25), and those living in unique environments may have special lipids (5). It is likely that *H. pylori* living in the gastric niche may have a distinct lipid composition or structure. The fatty acid composition of *H. pylori* has been studied extensively (9, 10, 19), and it has been shown that the cellular fatty acids 3-OH C_{16:0} and 3-OH C_{18:0} are unique to this organism. However, little is known about the polar lipid composition of *H. pylori*.

Therefore, we studied the lipid composition of *H. pylori* and detected three kinds of glycolipids, which were identified as cholesteryl glucosides (CGs). Additionally, the molecular structures of these CGs have been determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* NCTC 11638 was mainly used in this study. *H. pylori* ATCC 43504 and two clinical isolates of *H. pylori* obtained from Japan and Bangladesh were used for comparison. These were grown in brain heart infusion broth (Difco, Detroit, Mich.) supplemented with 5% horse serum. After a preculture was diluted 50-fold with fresh medium,

cultures were grown with shaking in sealed jars at 37°C for 2 days under a microaerophilic condition by using Anaeropack Campylo (Mitsubishi Gas Chemical, Tokyo, Japan). Almost all the bacteria grown under this condition were helical.

Staphylococcus aureus FDA 209P and *Escherichia coli* K-12 were also used in this study. These bacteria were grown under the same growth condition as *H. pylori*.

Extraction and purification of lipids. Bacteria were harvested and washed three times with physiological saline by centrifugation. Lipids were extracted from cell pellets three times with 20 volumes of a chloroform-methanol mixture (1:1 [vol/vol]). The lipid extracts were combined and purified by the method of Folch et al. (7) and used as total lipids.

Identification of lipids on 2D TLC. Total lipid was chromatographed on a Silica Gel G (Merck, Darmstadt, Germany) by two-dimensional thin-layer chromatography (2D TLC) plate with chloroform-methanol-distilled water (65:25:4 [vol/vol/vol]) as the first developing solvent system and chloroform-methanol-7 N NH₄OH (60:35:4.5 [vol/vol/vol]) as the second one. Each spot was characterized with the following stains: Dittmer's stain (6) for phospholipids, orcin-sulfuric acid stain (0.2% orcinol in 2 N H₂SO₄) for glycolipids, ninhydrin stain (17) for amino lipids, and Dragendorff stain (2) for lipids containing choline. Finally, the lipids on the TLC plate were identified by comparing their R_f values with those of authentic lipids by 2D TLC. The following lipids were used as authentic samples: cardiolipin (CL) (purified from *E. coli* and *S. aureus*), phosphatidyl glycerol (PG) (purified from *E. coli*, and synthetic dipalmitoyl [Sigma, St. Louis, Mo.]), phosphatidyl ethanolamine (PE) (purified from *E. coli*), phosphatidyl choline (PC) (from egg yolk; Sigma), phosphatidyl serine (PS) (from bovine brain; Sigma), sphingomyelin (from bovine brain; Sigma).

Fractionation of total lipid. Total lipid was fractionated by chromatography on a silicic acid column (Iatrobeads 6RS-8060; Iatron, Tokyo, Japan). Total lipids were dissolved in chloroform and applied to the column. Elutions were carried out with 10 column volumes of the following solvents: chloroform, chloroform-acetone (1:1 [vol/vol]), acetone, acetone-methanol (3:1 and 1:1 [vol/vol]), and methanol.

Analysis of phospholipids. The spots corresponding to phospholipids were scraped off the 2D TLC plate, and the phosphorus content was determined by the method of Kates et al. (18).

Major phospholipids (PE, PG, and cardiolipin) were purified from the 2D TLC plate by scraping off the spots corresponding to major phospholipids and extracting them from the scraped powder with chloroform-methanol (1:1). Purified phospholipids were used for analysis of fatty acids and for comparison with the constituents of glycolipids.

Analysis of glycolipids. Glycolipids (G-1, G-2, and G-3) were purified from the fractionated lipids as follows. The chloroform-acetone (1:1 [vol/vol]) fraction was used for purification of G-1 and G-2, and the acetone-methanol (3:1) fraction was used for G-3. Separation of each glycolipid from the fractionated lipids was carried out by preparative TLC on a precoated Silica Gel G 60 plate (5721; Merck) with chloroform-methanol-distilled water (70:30:5 [vol/vol/vol]). Each

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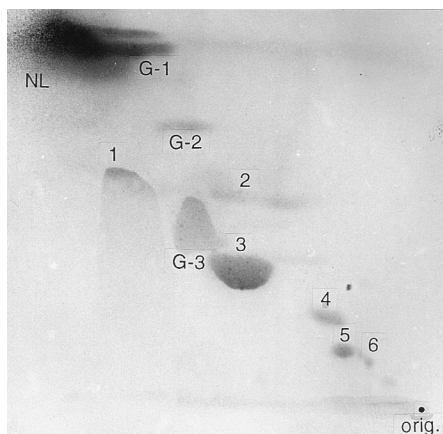


FIG. 1. 2D TLC of total lipid. NL, neutral lipids; G-1, G-2 and G-3, glycolipids (G-3 also contained phosphorus); 1 to 6, phospholipids CL, PG, PE, PC, PS, and sphingomyelin, respectively; orig., origin. The plate was stained with 30% sulfuric acids. The solvent systems used were chloroform-methanol-water (65:25:4) for the first direction and chloroform-methanol-7 N NH_4OH (60:30:4.5) for the second direction.

glycolipid was extracted from the TLC plate by the same method as for analysis of phospholipids.

For analysis of cholesterol, 1 mg of each purified glycolipid was methanolized with 1 ml of methanolic HCl (5%) at 100°C for 3 h. After cooling, the reaction mixtures were extracted three times with hexane. The hexane extracts were combined and dried in vacuo. The samples and authentic cholesterol were analyzed by TLC with petroleum ether-ether (9:1 [vol/vol]). The dried hexane extract, which contained liberated cholesterol, was redissolved in isopropanol. Cholesterol was determined quantitatively by the enzymatic method of Allain et al. (1), with a commercial kit (FREE CHOLESTEZYME-V555; Eiken Chemical, Tokyo, Japan).

For analysis of sugar, the remaining methanol phases of the methanolysis described above (analysis of cholesterol) were neutralized with silver carbonate. After the addition of acetic anhydride, the solutions were incubated at room temperature for 6 h, filtered with a filter paper, and dried in vacuo. The residues were trimethylsilylated with a mixture consisting of 0.2 ml of hexamethyldisilazane, 0.1 ml of trimethylchlorosilane, and 0.5 ml of pyridine. The trimethylsilylated methyl glycosides were analyzed by gas-liquid chromatography (model 263-70 chromatograph; Hitachi, Tokyo, Japan) on a column (3 mm [inside diameter] by 3 m) packed with 3% OV-1. Sugar was quantitatively determined directly from purified glycolipids by the method of Francois et al. (8), with an orcin-sulfuric acid reagent.

For determination of glycerol, the individual glycolipids were hydrolyzed with 2 N HCl at 125°C for 48 h. After cooling, methanol and chloroform were added to the solutions to a final methanol-chloroform-2 N HCl ratio of 10:10:9 (vol/vol/vol), and after adequate mixing, the chloroform phase was taken out. The extraction with chloroform and methanol was repeated three times, and the remaining aqueous-methanolic phase solution was dried in vacuo and dissolved in distilled water. This solution was used for the analysis of glycerol. Glycerol was determined quantitatively with the periodate-chromotropic acid reagent by the method of Renkonen (29).

Weak alkaline hydrolysis was done to determine the relationship among the glycolipids. Purified glycolipids, in chloroform-methanol (1:1 [vol/vol]), were adjusted to pH 12 with 1 N sodium methoxide in methanol and incubated at room temperature for 30 min. After neutralization with 1 N acetic acid in methanol, the reaction mixtures were analyzed by TLC on a precoated Silica Gel G 60 plate with a solvent mixture consisting of chloroform-methanol-distilled water (70:30:5 [vol/vol/vol]).

Analysis of fatty acids. Total lipid, major phospholipids (individual), and glycolipids (individual) were methanolized by methanolic HCl (5%) at 100°C for 3 h. After cooling, the reaction mixtures were diluted with 1/9 volume of water and extracted three times with hexane. Fatty acid methyl esters were analyzed in a gas-liquid chromatograph equipped with a flame ionization detector (model G-3000; Hitachi) on a fused-silica capillary column (0.53 mm [inside diameter] by 25 m) packed with OV-1. The column temperature was programmed to increase from 160 to 250°C at 7°C/min. Fatty acid methyl esters were identified by comparing their retention times with those of the authentic samples. For the quantitative determination, free fatty acids were liberated from purified glycolipids by hydrolysis as described above (determination of glycerol in analysis of glycolipids). After hydrolysis, extraction with chloroform and methanol was repeated three times. The chloroform-phase solutions were combined, dried in vacuo, and redissolved in isopropanol. This solution was used for the quantitative

analysis of fatty acids. Fatty acids were determined by the enzymatic method of Okabe et al. (26), with a commercial kit (NEFAZYME-S; Eiken Chemical).

Optical rotations and spectral analysis. Optical rotations were measured on a JASCO DIP-4 polarimeter in chloroform-methanol (1:1 [vol/vol]) at 20°C. ^1H nuclear magnetic resonance (^1H -NMR) spectra were recorded in CDCl_3 - CD_3OD (1:1 [vol/vol]) on a model VXR 500 (Varian, Palo Alto, Calif.) instrument (500 MHz for ^1H) at ambient temperature, and chemical shifts are given in δ (ppm) relative to those of tetramethylsilane. The ^1H - ^1H shift correlation spectroscopy and J -resolved 2D spectra were recorded by using the standard pulse sequences of the Varian recorder. The fast-atom bombardment-mass spectroscopy (FAB-MS) spectrum was recorded on a gas chromatograph-mass spectrometer (model GCMS 9020-DF; Shimadzu, Kyoto, Japan). The samples were dissolved in chloroform-methanol (1:1) at a concentration of ca. 6 mg/ml, and nitrobenzyl alcohol or glycerol was used as a matrix. The target surface was bombarded by a beam of energetic argon atoms at 5 keV and 5 mA.

Hemolytic activities of glycolipids (CGs) and total lipids. Each purified glycolipid (CG) in chloroform-methanol (1:1) was mixed with PC in chloroform-methanol (1:1), and the mixture was dried in vacuo. Phosphate-buffered saline (PBS; pH 7.4) was added to the sample at a concentration of 0.4 mg/ml. Liposomes were made by sonication for 30 s at 45°C in a bucket-type sonicator (model 41-4000; Branson, Stamford, Conn.). Liposomes of total lipids of *H. pylori*, *S. aureus*, and *E. coli* were made in the manner described above. The solution containing liposomes was mixed with an equal volume of 2% horse erythrocytes in PBS and incubated at 37°C overnight with gentle rotation. The sample was centrifuged at $800 \times g$ for 10 min. A 100-fold dilution of a lysis buffer containing 6% (wt/vol) sodium dodecyl sulfate and 7% (vol/vol) Triton X-100 in 0.03 M phosphate buffer (pH 7.2) was added to the pellet. The amount of hemoglobin was measured at 540 nm with a spectrophotometer (Hitachi model U-2000), and the number of remaining erythrocytes was calculated.

RESULTS

Qualitative analysis of each lipid. The total lipid extracts showed nine distinct spots on the 2D TLC plate (Fig. 1). Among them, three spots that were positive with orcin-sulfuric acid stain were characterized as glycolipids and tentatively named G-1, G-2, and G-3. Seven spots, including G-3, that were positive with Dittmer's stain were suggested to be phospholipids. These spots, except G-3, were numbered as shown in Fig. 1. Because G-3 was positive for both orcin-sulfuric acid stain and Dittmer's stain, it was regarded as a phosphoglycolipid. With ninhydrin stain, spots 3 and 5 were positive, indicating that they are phosphoaminolipids. Spots 4 and 6 were classified as lipids containing choline on the basis of the positive reaction with Dragendorff reagent. Finally, the lipids on the TLC plate were identified by comparing their R_f values with those of authentic lipids as follows: spot 1, CL; spot 2, PG;

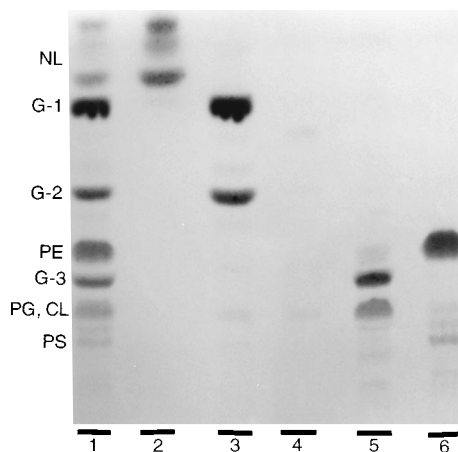


FIG. 2. One-dimensional TLC of fractionated lipids with silicic acid column chromatography. Lanes: 1, total lipid; 2, chloroform fraction (pass-through fraction); 3, chloroform-acetone (1:1) fraction; 4, acetone fraction; 5, acetone-methanol (3:1) fraction; 6, acetone-methanol (1:1) fraction. The plate was stained with 30% sulfuric acid. The solvent system used was chloroform-methanol-water (70:30:5). NL, neutral lipids.

TABLE 1. Phospholipid composition of *H. pylori*

Phospholipid	% Phosphorus
PE	57.7
CL	21.5
PG	12.6
PS	2.0
PC	1.6
Sphingomyelin	1.0
G-3 (phosphoglycolipid)	2.9

spot 3, PE; spot 4, PC; spot 5, PS; spot 6, sphingomyelin (data not shown).

Lipid composition. Total lipids were fractionated by chromatography on a silicic acid column. Fractionated lipids, chromatographed on a TLC plate, are shown in Fig. 2. The individual fractions that eluted with the solvents contained lipids mainly as follows: chloroform (neutral lipids), chloroform-acetone (1:1) (G-1 and G-2), acetone (none), acetone-methanol (3:1) (PG, CL, and G-3), acetone-methanol (1:1) (PE and PS).

The lipid composition was calculated from the data of the fractionated lipids. Total lipid (by weight) consisted of 6.0% neutral lipids (chloroform fraction), 20.6% glycolipids (G-1 and G-2) (chloroform-acetone [1:1 fraction]) and 73.4% phospholipids including G-3 (other fractions). Each glycolipid was purified from the fractionated lipids as described above. On the basis of the yield of each purified glycolipid by preparative TLC, the percentage of total glycolipids (G-1, G-2, and G-3) was shown to be about 25% (wt/wt) of the total lipids, and the ratio of G-1/G-2/G-3 was about 1:0.5:0.3 (wt/wt/wt). In other strains of *H. pylori* (ATCC 43504 and two clinical strains), all three kinds of glycolipids were also detected. The ratio between G-1 and G-2, however, varied, depending on the strains or culture lots. In *H. pylori* ATCC 43504, the content of G-1 was less than that of G-2 (data not shown).

In the total lipids of *S. aureus*, *E. coli*, and horse serum, supplement of the medium, no glycolipids with the same R_f values as those of *H. pylori* were detected by TLC.

The phospholipid composition is shown in Table 1. The major phospholipids (with the percentage of phosphorus given in parentheses) were PE (57.7%), CL (21.5%), and PG (12.6%). PS was detected at a concentration of 2.0%. The content of G-3 was 2.9% phospholipid.

The fatty acid compositions of the total lipid, major phospholipids, and glycolipids are shown in Table 2. The fatty acid compositions of the major phospholipids and G-3 were similar to that of the total lipid. However, only $C_{14:0}$ was found in G-1,

TABLE 2. Fatty acid composition of total lipid and each purified lipid

Lipid ^a	% Fatty acid					
	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{19:0}$ cyc ^b
TL	58.6	2.9	4.9	9.4	2.9	20.0
PE	47.7	1.7	2.5	11.8	1.7	33.6
PG	45.1	6.6	8.6	13.2	3.1	21.6
CL	51.2	6.5	6.7	8.7	2.8	20.6
G-1	98.5	Trace	Trace			
G-2						
G-3	43.4	2.5	2.7	16.9	1.0	32.5

^a TL, total lipid.

^b $C_{19:0}$ cyc, cyclopropane fatty acid.

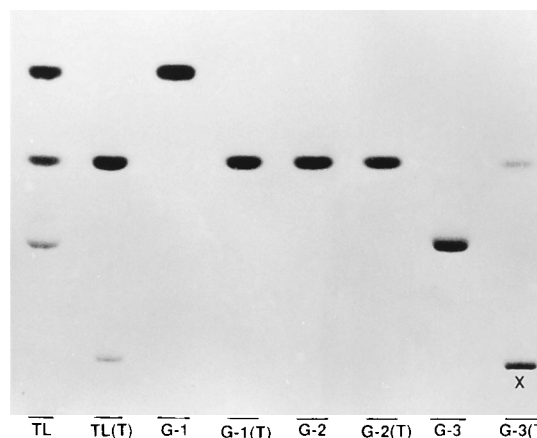


FIG. 3. One-dimensional TLC of purified glycolipids after weak alkaline hydrolysis. Weak alkaline hydrolysis was performed at pH 12 for 30 min with 1 N sodium methoxide. TL, total lipid; (T), after weak alkaline hydrolysis. The glycolipids were only visualized with orcin-sulfuric acid stain. The spots of X and G-3 were phosphoglycolipids which were also positive for Dittmer's stain. The solvent system used was chloroform-methanol-water (70:30:5).

and no fatty acid was detected in G-2. No 3-OH fatty acid was detected in the total lipid or in any of the isolated lipids.

Constituents of glycolipids. After methanolysis of purified glycolipids, total lipids, and purified PE, their hexane extracts were developed on a TLC plate. A spot with the same R_f value (0.44) as the authentic cholesterol and a positive reaction for sulfuric acid-acetic acid stain was detected in the extracts of purified glycolipids and total lipids but not in that of purified PE (data not shown). The residual sugars in the remaining methanol phase after methanolysis were analyzed as their trimethylsilylated derivatives by gas-liquid chromatography. Only glucose was detected in purified glycolipids (data not shown). On the basis of these data, the glycolipids were identified as CGs. Furthermore, they were found to be related to each other, because weak alkaline hydrolysis of G-1 and G-3 yielded G-2 as a product (Fig. 3). After the weak alkaline hydrolysis of G-3, a spot (X in Fig. 3) other than the spot of G-2 which was positive for the color reaction of phosphate (Dittmer's stain) was detected and named G-3X.

Quantitative analyses of the constituents of the identified CGs are summarized in Table 3. On the basis of the data of the quantitative analyses of the constituents, the weak alkaline hydrolysis (Fig. 3), and analyses of fatty acids (Table 2), it was inferred that G-2 contained glucose and cholesterol in a molar ratio of 1:1, G-1 contained the G-2 moiety and fatty acids ($C_{14:0}$) in a molar ratio of 1:1; and G-3 contained the G-2 moiety, fatty acids, glycerol, and phosphate in a molar ratio of 1:2:1:1.

Structures of G-2. G-2, $[\alpha]_D^{+67.5^\circ}$ ($c = 0.5$, chloroform-methanol [1:1]), was obtained as a white amorphous powder. Its FAB-MS spectrum showed an ion peak at m/z 571 (Fig. 4) assignable to a $[M + Na]^+$, which shifted to m/z 587 $[M + K]^+$

TABLE 3. Molar ratio of constituents of glycolipids

Glycolipid	Glucose	Cholesterol	Fatty acids	Glycerol	Phosphorus
G-1	1.00	1.02	0.86	0.02	ND ^a
G-2	1.00	0.97	0.04	0.04	ND
G-3	1.00	0.93	2.01	0.82	0.97

^a ND, not detected.

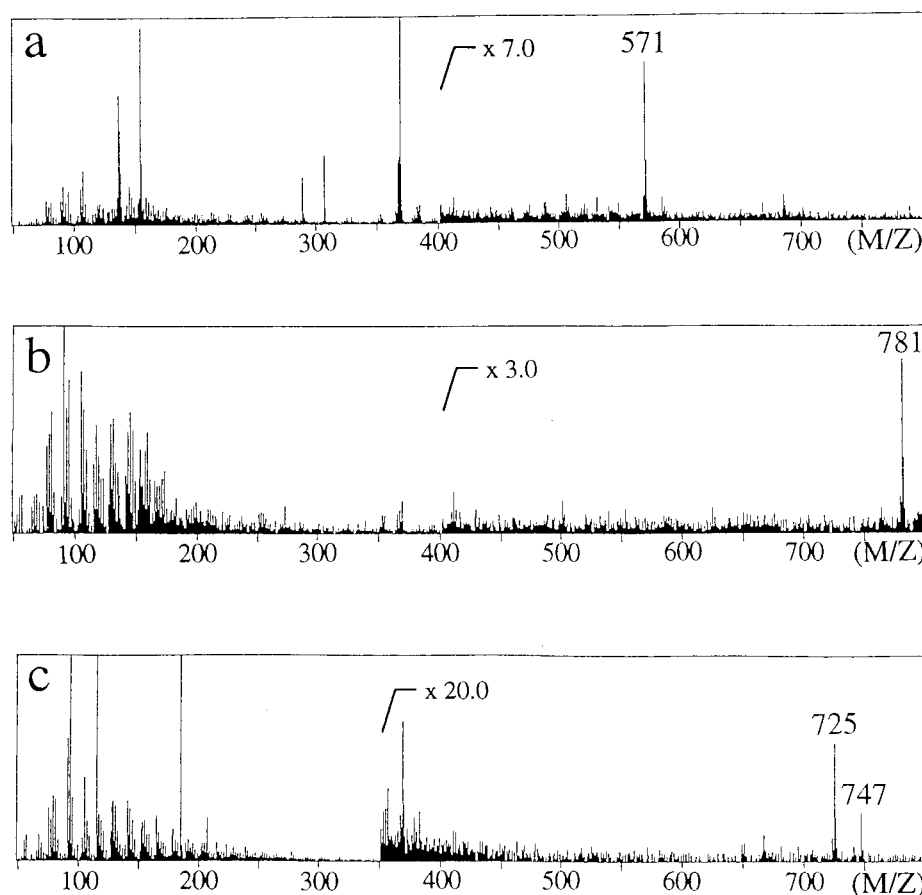


FIG. 4. FAB-MS spectra of G-1, G-2, and G-3X. (a) G-2 (nitrobenzyl alcohol matrix). An ion peak at m/z 571 was assigned as $[M + Na]^+$. (b) G-1 (nitrobenzyl alcohol matrix). An ion peak at m/z 571 was assigned as $[M + Na]^+$. (c) G-3X (glycerol matrix). Ion peaks at m/z 747 and 725 were ascribable to $[M + Na_2H]^+$ and $[M + Na]^+$, respectively.

with the addition of potassium chloride to the matrix agent. These data along with the production of only cholesterol and glucose by acid hydrolysis with 2 N HCl (Table 3) indicated the molecular formula of G-2 to be $C_{33}H_{56}O_6$ (molecular weight of 548). The glucose residue in G-2 was shown to adopt a C1 conformation by large coupling constants between vicinal protons ($J_{2,3} = J_{3,4} = J_{4,5} = 10$ Hz) in the 500-MHz 1H -NMR spectrum ($CDCl_3$ - CD_3OD) (Table 4), which were assigned by 1H - 1H shift correlation spectroscopy. A small coupling constant ($J = 4$ Hz) of the doublet (δ 4.98) due to an anomeric proton indicated an α -glycosidic linkage. Therefore, G-2 was characterized as cholesterol 3- O - α -D-glucopyranoside (Fig. 5).

Structures of G-1. G-1, $[\alpha]_D +43.8^\circ$ ($c = 0.5$, chloroform), showed a prominent peak at m/z 781 $[M + Na]^+$ by FAB-MS (Fig. 4). The 1H -NMR spectrum of G-1 was similar to that of G-2. A distinguishing spectral feature between G-1 and G-2 was the appearance of extra signals due to a primary methyl group and methylene groups at δ 0.86 (3H, t, $J = 6.5$ Hz), 1.30 (22H, s), and 2.33 (2H, t, $J = 6.5$ Hz) in G-1, which indicated the presence of a C_{14} acyl group in G-1. Remarkable downfield shifts of the glucose methylene proton signals (H-6'; δ 4.42 [dd, $J = 5$ and 12 Hz] and 4.26 [dd, $J = 2$ and 12 Hz]) relative to those (δ 3.77 [dd, $J = 4.5$ and 12 Hz] and 3.81 [dd, $J = 3$ and 12 Hz]) of G-2 were also observed, indicating that the C_{14} acyl group in G-1 is at O -6' of the glucose residue (Table 4). The α -glycosidic linkage in G-1 was evident by the coupling constant ($J = 4$ Hz) of the doublet (δ 4.98) due to an anomeric

proton, similar to that of G-2. On the basis of these data, G-1 was concluded to be cholesterol 3- O -(6'- O -tetradecanoyl)- α -D-glucopyranoside (Fig. 5), which was consistent with the result of quantitative analysis of the constituents of G-1 (Table 3).

Structures of G-3. A product (G-3X) of G-3, $[\alpha]_D +24.6^\circ$ ($c = 1.0$, chloroform-methanol [1:1]), which is positive for the color reaction for phosphate (Dittmer's stain) was obtained by weak alkaline hydrolysis (Fig. 3). The 1H -NMR spectrum ($CDCl_3$ - CD_3OD) of G-3X exhibited, in addition to signals similar to those of G-2, a methine (δ 3.81, q, $J = 5.5$ Hz) and two methylene proton signals in the range of 3.9 to 4.2 ppm, which are assigned to a glycerol moiety in G-3X. The J -resolved 2D spectrum of G-3X revealed that each proton signal of glycerol is duplicated (data not shown). This phenomenon may be explained by rotational isomerism arising from steric hindrance around the glycerol moiety.

These data, coupled with the FAB-MS data (glycerol matrix: m/z 747 and 725 in Fig. 4 ascribable to $[M + Na_2H]^+$ and $[M + Na]^+$, respectively) corresponding to the molecular formula $C_{36}H_{62}O_{11}P$ (molecular weight of 702), clearly indicated that the product (G-3X) is a glyceryl phosphate of G-2. Acetylation of G-3X gave a pentaacetate (G-3X-Ac) which showed an $[M + Na]^+$ ion peak at m/z 935 in the FAB-MS spectrum (data not shown), and five acetoxyl methyl signals at δ 2.02 to 2.10 in the 1H -NMR spectrum. On comparison of the glucose proton signals between G-3X and its acetate (G-3X-Ac), the glucose H-2', H-3', and H-4' signals in G-3X-Ac showed large down-

TABLE 4. ^1H -NMR data for glucose and glycerol moieties of glycolipids^a

Moiety	^1H -NMR result for:			
	G-1	G-2	G-3X	G-3X-Ac
Glucose				
H-1'	4.98 d ($J = 4$)	4.98 d ($J = 4$)	4.97 d ($J = 4$)	5.24 d ($J = 3.5$)
H-2'	3.46 dd ($J = 4, 10$)	3.41 dd ($J = 4, 10$)	3.41 dd ($J = 4, 10$)	4.80 dd ($J = 3.5, 10$)
H-3'	3.70 t ($J = 10$)	3.67 t ($J = 10$)	3.67 t ($J = 10$)	5.47 t ($J = 10$)
H-4'	3.31 t ($J = 10$)	3.39 t ($J = 10$)	3.49 t ($J = 10$)	5.12 t ($J = 10$)
H-5'	3.84 ddd ($J = 2.5, 10$)	3.69 ddd ($J = 3, 4.5, 10$)	3.77 ddd ($J = 4, 6, 10$)	4.10 br.d ($J = 10$)
H-6'	4.26 dd ($J = 2, 12$) 4.42 dd ($J = 5, 12$)	3.77 dd ($J = 4.5, 12$) 3.81 dd ($J = 3, 12$)	3.66 dd ($J = 6, 11$) 3.60 dd ($J = 4, 11$)	4.00 m 3.96 m
Glycerol ^b				
H-1''			4.08 dd ($J = 5.5, 11$) 4.07 dd ($J = 5.5, 11$)	4.01 m 3.96 m
H-2''			3.81 q ($J = 5.5$) 3.80 q ($J = 5.5$)	5.22 m
H-3''			3.99 dd ($J = 5.5, 11$) 3.98 dd ($J = 5.5, 11$)	4.42 dd ($J = 3.5, 12$) 4.21 dd ($J = 6, 12$)

^a Assignments were confirmed by ^1H - ^1H shift correlation spectroscopy (500 MHz, CDCl_3 - CD_3OD [1:1]; J is expressed in hertz).

^b Each proton signal was duplicated, probably because of rotational isomerism.

field shifts of 1.4 to 1.8 ppm from the corresponding signals of G-3X (Table 4), while the methylene proton signals (H-6'; δ 3.96 and 4.00) showed only small shifts (ca. 0.3 ppm). A similar large downfield shift by 1.4 ppm was also observed for a methine proton (H-2'') of glycerol. In addition, double doublets (δ 4.42 and 4.21) due to one of the methylene groups of glycerol were shifted to a lower field than that (δ 3.99 and 3.93) of G-3X, whereas the other methylene protons were almost unaffected on acetylation (Table 4). These data indicated that the acetyl groups were introduced into O-2', O-3', and O-4' of the glucose core and O-2'' and O-3'' of the glycerol residue on acetylation of G-3X. The CG and glycerol moieties in G-3X were thus established to be connected through phosphate linkages between O-6' of glucose and O-1'' of glycerol (Fig. 5).

Although the signals in the ^1H -NMR spectrum of G-3, which contained a mixture of fatty acids (Table 2), could not be definitely assigned because of remarkable broadening for each proton, two signals at δ 5.01 (1H) and 4.35 (2H), which were observed along with H-6 (δ 5.30) and H-1' (5.20) in the low field region, were respectively attributable to H-2'' and H-3'' of an acylated glycerol moiety. G-3 was thus regarded as a mixture of fatty acid esters of G-3X, in which fatty acids are bound to the glycerol hydroxyl groups. Therefore, the most plausible structure of G-3 is represented by the formula shown in Fig. 6.

Hemolytic activities of each glycolipid (CG). We tried to make liposomes using only the glycolipids (CGs). However, in this case only G-3 was dispersed in PBS by sonication; G-1 and G-2 were not dispersed. The hemolytic activity of each CG was determined with liposomes made from lipid mixtures of each CG and PC. In the liposomes containing 50% CGs, all of the CGs demonstrated greater hemolytic activities than did the liposomes containing PC only, and among the CGs G-3 showed the strongest activity (Table 5). In the liposomes containing 14% G-1, 7% G-2, and 4% G-3, G-3 showed the strongest hemolytic activity (Table 5). Furthermore, the liposome

made from the total lipid of *H. pylori* showed higher levels of hemolytic activity (49%) than those of *E. coli* (29%) and *S. aureus* (22%).

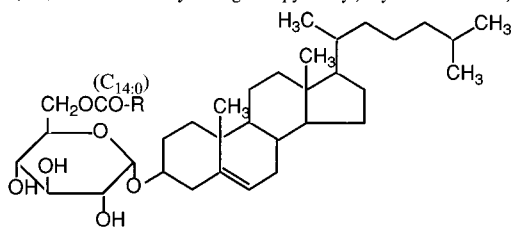
DISCUSSION

The polar lipid composition of the *Helicobacter* genus has not been investigated. This is the first report on the polar lipid composition of *H. pylori*. *H. pylori* NCTC 11638, having almost all cells in the helical form, was used in this study. Neutral lipids were not observed in significant proportion. Among the phospholipids, PE was dominant, and other major lipids were CL and PG; this is similar to many other gram-negative bacteria. PS was detected at a concentration of 2.0% of the total phospholipids. Small amounts of PC and sphingomyelin were detected, but the possibility that they were transferred from the culture medium containing horse serum could not be ruled out.

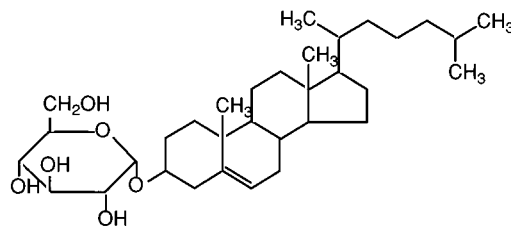
The fatty acid composition of *H. pylori* has been well demonstrated in many studies (9, 10, 19). Among cellular fatty acids, *H. pylori* has 3-hydroxy hexadecanoic acid (3-OH $\text{C}_{16:0}$) and 3-hydroxy octadecanoic acid (3-OH $\text{C}_{18:0}$), which are unique to this organism (19); these 3-OH fatty acids are, however, derived from lipopolysaccharide (9). In chloroform-methanol-extractable lipids they are absent, and the major fatty acids are tetradecanoic acid ($\text{C}_{14:0}$) and 19-carbon cyclopropane fatty acid ($\text{C}_{19:0}$ cyc). In our study, the major fatty acids of the total lipid were $\text{C}_{14:0}$ and $\text{C}_{19:0}$ cyc. 3-OH fatty acids could not be detected in the total lipid.

We found three kinds of glycolipids (G-1, G-2, and G-3) in the 2D TLC of the total lipid from *H. pylori*. Glycolipid accumulation was quite high and contributed about 25% of the total lipid. Because no glycolipids with the same R_f values by TLC as those of *H. pylori* were detected in the lipids of the horse serum employed for culture and of *S. aureus* and *E. coli*

G-1: Cholesteryl-6-*O*-tetradecanoyl- α -D-glucopyranoside
 { 3-(6-*O*-Tetradecanoyl- α -D-glucopyranosyl)oxy-cholest-5-ene }



G-2: Cholesteryl- α -D-glucopyranoside
 { 3-(α -D-Glucopyranosyloxy)-cholest-5-ene }



G-3X (deacylated G-3):
 Cholesteryl-6-*O*-phosphoryl- α -D-glucopyranoside
 { 3-[6-*O*-(3-phosphoryl)- α -D-glucopyranosyl]oxy-cholest-5-ene }

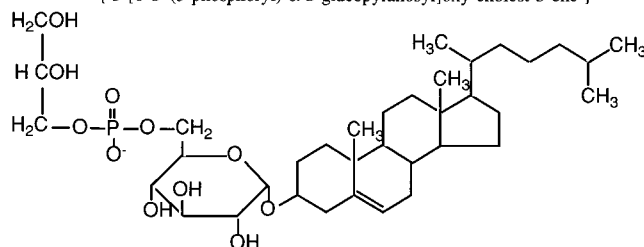


FIG. 5. Determined molecular structures of G-1, G-2, and G-3X.

grown in the same growth condition as that of *H. pylori*, these glycolipids were synthesized de novo in *H. pylori*. From the results of several experiments, these glycolipids were identified as CGs, and their molecular structures (G-1, G-2, and deacylated G-3) were determined (Fig. 5).

Deacylated G-3 (G-3X) was used in ^1H -NMR spectroscopy, because G-3, which has many kinds of fatty acids as constituents, did not exhibit clear signals. The acylated positions of G-3X remained unknown. However, the ^1H -NMR spectral data of G-3 in comparison with that of G-3X suggested that fatty acids are bound to the glycerol hydroxyl groups in G-3X, and these hydroxyl groups were easily esterified. Therefore, the

Cholesteryl-6-*O*-phosphatidyl- α -D-glucopyranoside
 { 3-[6-*O*-(3-phosphatidyl)- α -D-glucopyranosyl]oxy-cholest-5-ene }

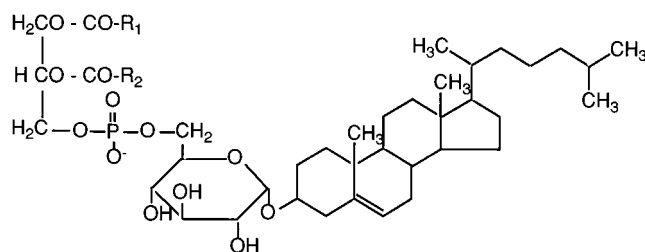


FIG. 6. Most plausible structure of G-3.

TABLE 5. Hemolytic activities of liposomes containing CGs against horse erythrocytes^a

Liposome composition	Hemolysis (%) of:				
	G-1	G-2	G-3	PC only	Horse serum
50% CGs ^b	28	38	75	18	12
CGs (as present in the bacterium) ^c	30	29	46	20	10

^a The liposomes were mixed with 1% (final concentration) horse erythrocytes and incubated with gentle rotation at 37°C overnight.

^b The liposomes contained each CG and PC (1:1 [wt/wt]) at a final concentration of 0.2 mg/ml in PBS (pH 7.4).

^c The liposomes contained (by weight) 14% G-1, 7% G-2, and 4% G-3, with PC composing the remaining percentage.

most plausible structure of G-3 is represented by the formula in Fig. 6.

Steryl glycosides are found in many plants (34). CGs are also found in plants (3) and fungi (16), but they are very rare in animals and bacteria. In bacteria, CGs have been reported only in *Acholeplasma* spp. (22, 30), *Mycoplasma gallinarum* (30, 33), *Spiroplasma citri* (28), and *Borrelia hermsii* (20). Most of the glucose residues of the sterol-based compounds are β -linked pyranoses, while the α anomer has been reported in *Acholeplasma laidlawii* and *Acholeplasma axanthum* (22). The anomeric configuration in the CGs of *H. pylori* was α . Further, acylated derivatives of CGs like G-1 are found in *A. axanthum* and *B. hermsii* (22, 28). No phosphate-linked CG like cholesteryl-6-*O*-phosphatidyl- α -D-glucopyranoside (G-3) has yet been found either in prokaryotes or in eukaryotes. We also investigated the lipid components in other strains of *H. pylori* (ATCC 43504 and two clinical strains). In all three strains, three kinds of CGs were detected. Therefore, the presence of the CGs is a unique feature of *H. pylori*.

For the synthesis of CGs, cholesterol is needed. However, with the exception of a stable L-form of *S. aureus* (11), bacteria cannot synthesize cholesterol. Most of the bacteria having CGs require cholesterol or serum for their growth. In *Acholeplasma*, which do not require cholesterol, CGs are found only when the organisms are supplied with exogenous cholesterol (30). *H. pylori* is usually grown in a medium supplemented with blood or serum, as in this study, but the organism can grow in media containing dimethyl β -cyclodextrin and no serum (27). Therefore, like *Acholeplasma*, *H. pylori* may synthesize CGs only when the growth medium contains cholesterol. However, *H. pylori* growing in the human gastric mucosa probably also synthesizes CGs, since a large amount of cholesterol is likely to be available in that environment. Besides cholesterol, glucose is also required for the synthesis of CGs. Although *H. pylori* has no fermentative pathways for saccharides, glucose utilization by the organism has been reported (23, 31). In a recent report, it was suggested that the organism contains unknown glucosides (31). The significant content of CGs in *H. pylori* adds a new facet to the physiology and biochemistry, especially the cholesterol and glucose metabolism, of the organism.

H. pylori colonizes the human gastric mucosa, and infection by the organism is recognized as a primary cause of active chronic gastritis (4). In the gastric mucosa, most of the bacteria are free swimming, but some adhere closely to the surface of the mucus neck cells of the antrum (12). The close adhesion and/or destruction of the organism may expose the host cells to the CGs. It has been observed that steryl glycosides possess hemolytic properties (32). In this work, the CG of *H. pylori* showed hemolytic activities. The liposome of G-3 showed particularly high levels of activity, even at a 4% concentration (as

was present in the bacterium). Furthermore, the liposome made from the total lipid of *H. pylori* showed hemolytic activity. On the basis of these data, CGs may cause damage to the gastric mucosal cells in persistent *H. pylori* infection.

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